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Triggering of Natural Killer Cells by the Costimulatory Molecule CD80 (B7-1)

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Summary

NK cell–mediated cytotoxicity is influenced by triggering as well as inhibitory signals. The identification of inhibitory signals provided by MHC class I molecules has recently attracted significant attention. Much less is known about putative triggering signals. Using purified populations of mouse NK cells, we demonstrate that the CD80 (B7-1) gene product functions as a triggering signal for NK cell–mediated cytotoxicity. The strength of this response is such that it overrides the protection mediated by MHC class I molecules. Triggering of mouse NK cells by B7-1 occurred even in the absence of CD28 and could not be blocked by either anti-CD28 or anti-CTLA-4 antibodies. NK cells may thus, at least in part, use receptors other than CD28 and CTLA-4 in their interaction with B7-1. Furthermore, we demonstrate that bone marrow–derived macrophages and dendritic cells are highly susceptible to lysis by autologous NK cells.

Introduction

Natural killer (NK) cell–mediated cytotoxicity is influenced by triggering as well as inhibitory signals (Gumperz and Parham, 1995; Lanier and Philips, 1996; Raulet, 1996). Recent studies have demonstrated that target cell major histocompatibility complex (MHC) class I molecules can turn off NK cells (Ljunggren and Kärre, 1990; Kärre, 1995), by delivering a negative signal to specific MHC class I–binding receptors (Karlhofer et al., 1992; Yokoyama and Seaman, 1993). The latter include members of the Ly-49 gene family in mouse and the killer cell inhibitor receptor (KIR) gene family in humans (Gumperz and Parham, 1995; Lanier and Philips, 1996; Raulet, 1996). On the other hand, triggering receptors, such as members of the NKR-P1 family (Yokoyama and Seaman, 1993), may receive stimulatory signals from as yet unidentified ligands. The general notion holds that NK cells must be triggered by any of a set of distinct target cell ligands, but that all of these signals can be overruled by MHC class I–mediated inhibition (e.g., see Correa et al., 1994).

The CD80 (B7-1) gene product, and its counterreceptors CD28 and CTLA-4, have been extensively characterized in recent years in their delivery of costimulatory signals to naive T cells (Linsley and Ledbetter, 1993; Allison, 1994; Sharpe, 1995). B7-1 is expressed at high levels on hematopoietic cells such as activated macrophages and dendritic cells. Recent studies have indicated that NK cells may be affected, indirectly or directly, by costimulatory molecules. Human adult NK cells are normally CD28 negative. However, a human $CD28^+$ NK cell leukemia YT2C2 cell line (or variants of this line) spontaneously lysed human as well as mouse cell lines expressing B7-1 (Azuma et al., 1992; Montel et al., 1995a). Furthermore, rejection responses against murine B7-1–transfected tumor cell lines were likely to involve an NK cell component (Cavallo et al., 1995; Geldhof et al., 1995; Wu et al., 1995; Yeh et al., 1995), and in two of these studies the B7-1-transfected tumor cell lines examined were killed by splenocytes in vitro at higher levels than corresponding wild-type cell lines (Geldhof et al., 1995; Yeh et al., 1995). An additional potential connection between B7-1 and NK cells is suggested by the observation that poly I:C–activated splenocytes could suppress antigen-presenting cells (APC) in mixed lymphocyte cultures (Gilbertson et al., 1986).

In the present study, we have directly addressed the ability of the costimulatory molecule B7-1 to trigger NK cell–mediated lysis. We demonstrate that MHC class I–positive NK cell–resistant targets become sensitive to NK cells after transfection of B7-1. These results suggest that the costimulatory molecule B7-1 functions as a triggering molecule for NK cell–mediated cytotoxicity in vitro and that the strength of this triggering response is such that it overrides the MHC class I–mediated protection against NK cell–mediated lysis. In addition, activated macrophages and dendritic cells, characterized by their expression of costimulatory molecules, were highly susceptible to NK cell–mediated lysis. Triggering of mouse NK cells by B7-1-transfected tumor cell lines and by activated APC occurred even in the absence of CD28 and could not be blocked by anti-CD28 and CTLA-4 antibodies. These data are discussed in relation to events involved in the triggering and inhibition of NK cell–mediated lysis.

Results

Purified NK Cells Are Triggered by B7-1-Expressing Tumor Cell Lines

C57BL/6 (B6)-derived lymphokine-activated killer (LAK) cells or Tilorone-activated splenocytes do not efficiently kill MHC class I–positive EL-4 cells. In contrast, similar effector cells readily killed EL-4 cells transfected with B7-1 (EL-4 B7-1), while sparing vector-transfected or untransfected control cells (Figure 1A; data not shown). These results were not a specific property of B7-1-transfected EL-4 cells. The MHC class I–positive RMA B7-1 and P815 B7-1 cell lines, respectively, were also highly sensitive to LAK cell lysis, while nontransfected wild-type cells were resistant to lysis (Figure 1B; data not shown for P815 B7-1). The lysis of EL-4 B7-1 and RMA B7-1 could be inhibited by addition of CTLA-4Ig, demonstrating that B7-1 was the triggering signal for target cell lysis and that the effect was not an indirect consequence of B7-1 expression (Figure 2).

It was important to verify that the effector cells within the LAK population, triggered by B7-1 expression on the tumor cell lines, fulfilled the criteria of being phenotypically normal NK cells. It was also important to assess

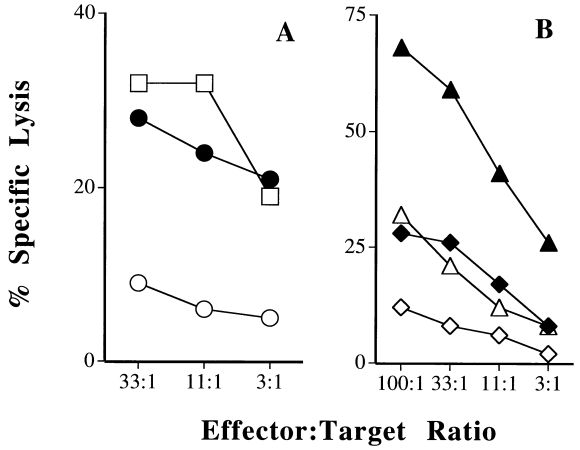

Figure 1. LAK Cell Lysis of MHC Class I–Positive and MHC Class I–Deficient Tumor Cell Lines Transfected with B7-1

(A) Target cells were as follows: EL-4 (open circles), EL-4 B7-1 (closed circles), and C4.4-25$^-$ (β2m$^-$ variant of EL-4; open squares). (B) Target cells were as follows: RMA (open diamonds), RMA B7-1 (closed diamonds), RMA-S (TAP2$^-$ variant of RMA; open triangles), and RMA-S B7-1 (closed triangles).

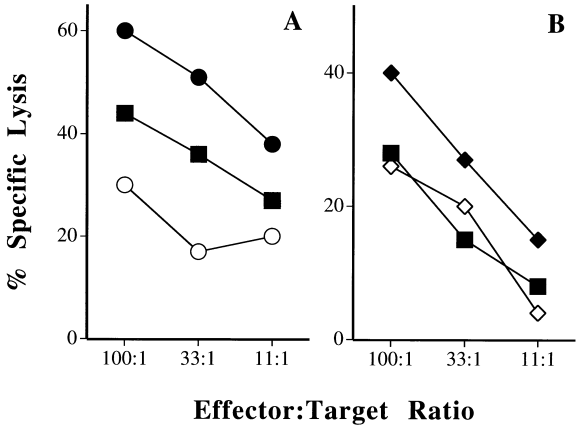

Figure 2. Lysis of EL-4 and RMA Tumor Cells Transfected with B7-1 Is Inhibited by CTLA-4Ig

(A) Target cells were as follows: EL-4 (open circles), EL-4 B7-1 (closed circles), and EL4-4 B7-1 and CTLA-4Ig (closed squares). (B) Target cells were as follows: RMA (open diamonds), RMA B7-1 (closed diamonds), and RMA B7-1 and CTLA-4Ig (closed squares).

whether triggering by B7-1 was a property of a subpopulation of NK cells or a more general property of all NK cells. Since the LAK cell cultures could be a potential source of cytotoxic T cells activated by IL-2, we first sorted the LAK cell cultures into NK1.1$^+$ and CD8$^+$ populations. All cytotoxic activity toward EL-4 B7-1 resided in the NK1.1$^+$ population of the LAK cells (Table 1). Similar effectors also preferentially killed the MHC class I–deficient EL-4 subline C4.4-25$^-$ as well as the standard NK target cell line YAC-1 (Table 1). A theoretical possibility was that all killing of B7-1-transfected target cells was mediated by a small fraction of NK1.1$^+$ T cells. We therefore sorted out NK1.1$^+$ TCR$\alpha\beta^-$ effectors. The NK1.1$^+$/TCR$\alpha\beta^-$ population was found to lyse EL-4 B7-1, but not EL-4 (data not shown). We then further subfractionated the NK1.1$^+$ population of LAK cells into NK1.1$^+$/Ly-49A$^+$, NK1.1$^+$/Ly-49A$^-$, NK1.1$^+$/Ly-49C$^+$, and NK1.1$^+$/Ly-49C$^-$ subpopulations. All four subpopulations of NK cells preferentially killed the B7-1-transfected EL-4 target cells over control EL-4 cells (Figure 3), indicating that the triggering of NK cells by B7-1 might be a more general property of NK cells.

NK Cell Triggering by B7-1 Overrides the Protection Mediated by MHC Class I Molecules

EL-4 and RMA cells are normally protected from NK cell lysis by virtue of their high constitutive levels of MHC class I expression (Ljunggren and Kärre, 1985; Kärre et al., 1986). Transfection of the B7-1 gene into these cell lines did not alter their levels of MHC class I expression as determined by FACS analysis (data not shown). The ability of NK cells to kill B7-1-transfected EL-4 and RMA cells demonstrated that the protection mediated by MHC class I expression could be overridden by the expression of B7-1. Indeed, B7-1-transfected EL-4 and RMA cells were rendered as sensitive to NK cell–mediated lysis as MHC class I–deficient variants of the EL-4 and RMA cell lines, respectively (Figure 1). Transfecting MHC class I–deficient RMA-S cells with B7-1 further potentiated the NK sensitivity of this cell line (Figure 1). This indicated that, even though the expression of B7-1 could override the protection imposed by MHC class I expression, MHC class I molecules were still able to offer some degree of protection (Figure 1).

In the studies of the NK cell subpopulations, we observed that the EL-4 as well as EL-4 B7-1 cell lines were killed at slightly reduced levels by NK1.1$^+$/Ly-49C$^+$ cells compared with the levels of lysis observed for the NK1.1$^+$/Ly-49C$^-$ subpopulation (Figure 3). This result is interesting in relation to the notion of Ly-49C being an inhibitory receptor for H-2K^b (Yu et al., 1996), since it provides additional support for the idea that B7-1 is able to override the protection mediated by MHC class I expression. However, on the other hand, it also indicates that MHC class I molecules can confer some level of protection from NK cell–mediated lysis despite B7-1 expression on the target cell.

Table 1. NK1.1$^+$ LAK Cells Kill B7-1-Transfected EL-4 Cells

	NK1.1$^+$ LAK				CD8$^+$ LAK			
Effector: Target Ratio	EL-4	EL-4 B7-1	C4.4-25$^-$	YAC-1	EL-4	EL-4 B7-1	C4.4-25$^-$	YAC-1
30:1	10	46	33	77	0	2	0	12
10:1	0	42	15	51	0	0	0	11
3:1	0	35	12	33	0	0	0	4
1:1	0	22	5	22	0	1	0	3

C4.4-25$^-$ is a β2m$^-$ mutant of EL-4. Results are expressed as percent specific lysis.

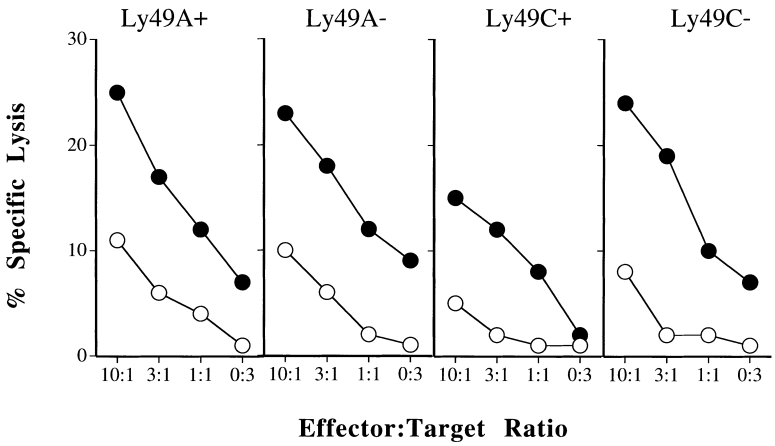

Figure 3. Lysis of EL-4 B7-1 Cells Is a Property of Several Defined Subsets of NK1.1$^+$ Cells

NK1.1$^+$ effectors were purified from LAK cell cultures and further subfractionated into NK1.1$^+$/Ly-49$^+$, NK1.1$^+$/Ly-49A$^-$, NK1.1$^+$/Ly-49C$^+$, and NK1.1$^+$/Ly-49C$^-$ populations. Target cells were EL-4 (open circles) and EL-4 B7-1 (closed circles).

NK Cell–Mediated Killing of B7-1-Transfected Tumor Cell Lines Occurs in the Absence of CD28 and Cannot Be Blocked by Antibodies against CD28 and CTLA-4

Recognition of B7-1 by T cells is mediated by the receptors CD28 and CTLA-4 (Linsley and Ledbetter, 1993; Allison, 1994; Sharpe, 1995). To address the role of CD28 on NK cells with respect to the triggering effects imposed by B7-1 expressed on tumor targets, we generated LAK effectors from CD28$^{-/-}$ mice. Effector cells from such mice killed EL-4 B7-1 targets at a level equivalent to effectors from wild-type (B6) mice (Table 2). Although this result did not exclude a role for CD28 on NK cells from wild-type mice, it suggested that receptors other than CD28 on NK cells might be capable of interacting with B7-1. To address further the role of CD28, as well as that of CTLA-4, in B7-1 recognition by NK cells, we preincubated LAK cells from B6 mice with anti-CD28 or anti-CTLA-4 antibodies (0.4–50 μg/ml). These experiments did not reveal any significant effects by either anti-CD28 or anti-CTLA-4 antibodies on the recognition of B7-1$^+$ targets (data not shown). Interestingly, these results indicate that NK cells may use receptors other than CD28 and CTLA-4 in interactions with B7-1 molecules.

NK Cell–Mediated Killing of B7-1-Expressing Targets Is Perforin Dependent

Studies of the human leukemic NK cell line YT2C2 (or variants of it) have suggested that cytotoxicity by this clone can be mediated by Fas–FasL interactions (Montel et al., 1995b; Roger et al., 1996). In contrast, NK cell–mediated cytotoxicity in the mouse has been demonstrated to be dependent on perforin (Kägi et al., 1994). To address whether mouse NK cells can use the Fas–FasL pathway, or pathways other than perforin-mediated killing, upon interaction with B7-1-transfected targets, we prepared LAK cells from perforin$^{-/-}$ mice. When such effectors were used, all killing capacity was abolished, strongly suggesting that the triggering of NK cells by B7-1 in the mouse leads to cytotoxicity mediated by perforin (Table 2).

NK Cells Lyse Bone Marrow–Derived Macrophages and Dendritic Cells

NK cell–mediated recognition of target cells expressing costimulatory molecules such as B7-1 implies that cells normally expressing such molecules, e.g., activated APC, may be targeted by NK cells. Indeed, autologous bone marrow–derived macrophages as well as dendritic cells were found to be highly susceptible to LAK cell–mediated lysis (Figure 4). In contrast with the results obtained with the macrophages or dendritic cell preparations, autologous concanavalin A (ConA)-activated T cell blasts were resistant to LAK cells. In contrast with bone marrow–derived macrophages, resting peritoneal macrophages were not lysed by similar LAK cells (data not shown). Macrophages and dendritic cells from MHC class I–deficient mice were found to be killed even more efficiently than similar cells from wild-type mice (Figure

Table 2. LAK Cell Lysis of EL-4 B7-1 Is Perforin Dependent, but Does Not Require CD28

	EL-4			EL-4 B7-1			YAC-1		
Effector: Target Ratio	B6	CD28$^{-/-}$	Perforin$^{-/-}$	B6	CD28$^{-/-}$	Perforin$^{-/-}$	B6	CD28$^{-/-}$	Perforin$^{-/-}$
Experiment 1									
100:1	27	24	0	67	56	1	56	65	4
33:1	21	22	0	58	49	1	50	46	4
11:1	20	11	0	56	41	0	48	39	3
3:1	13	8	0	46	23	0	32	18	1
Experiment 2									
100:1	11	9	0	37	34	3	70	72	9
33:1	9	6	0	38	30	0	64	61	8
11:1	3	4	0	34	25	0	62	62	11
3:1	1	2	0	27	17	1	45	48	13

Results are expressed as percent specific lysis.

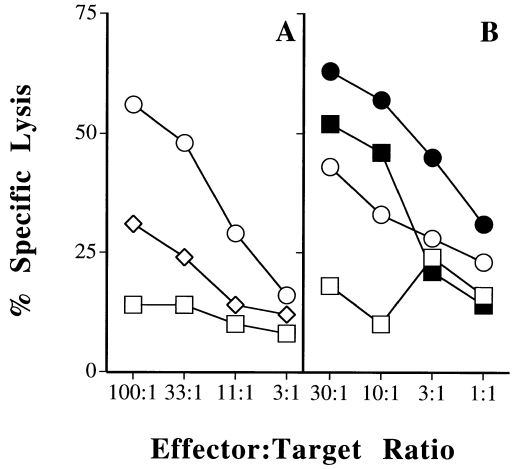

Figure 4. Bone Marrow–Derived Macrophages and Dendritic Cells Are Susceptible to LAK Cell Lysis

Bone marrow–derived macrophages and dendritic cells, as well as control ConA-activated T cell blasts, were tested for susceptibility to LAK cell–mediated lysis.

(A) Target cells were as follows: bone marrow–derived macrophages (open diamonds) and dendritic cells (open circles), as well as ConA-activated T cell blasts from B6 mice (open squares).

(B) Target cells were as follows: bone marrow–derived dendritic cells from B6 (open circles) and $\beta 2m^{-/-}$ mice (closed circles), as well as ConA-activated T cell blasts from B6 (open squares) and $\beta 2m^{-/-}$ mice (closed squares).

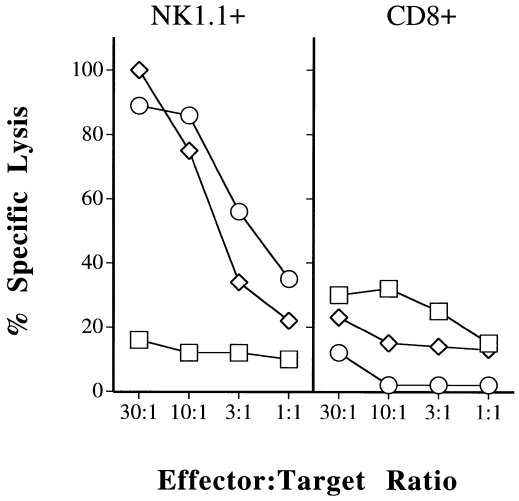

Figure 5. Lysis of Bone Marrow–Derived Macrophages and Dendritic Cells Is a Property of NK1.1$^+$ Cells

LAK effectors were sorted into NK1.1$^+$ and CD8$^+$ populations and used as effectors against bone marrow–derived macrophages (open diamonds) and dendritic cells (open circles), as well as ConA-activated T cell blasts (open squares) from B6 mice.

4; data not shown), analogous to MHC class I–deficient B7-1-transfected tumor cell lines (Figure 1).

As with B7-1-transfected tumor targets, all cytotoxicity against the bone marrow–derived macrophages and dendritic cells resided in the NK1.1$^+$ population of LAK cells (Figure 5). Blocking studies with CTLA-4Ig failed to block completely the killing of the bone marrow–derived macrophages or dendritic cells (data not shown). This observation indicates that molecules on these cells other than B7-1 and B7-2 may be involved in NK cell triggering, yet the results do not exclude a role for B7-1, B7-2, or both. LAK cell–mediated lysis of both macrophages and dendritic cells did not require CD28, as demonstrated using LAK cells from CD28$^{-/-}$ mice. As observed for B7-1-transfected tumor cell lines, LAK cell lysis of the macrophages and dendritic cells could not be blocked by antibodies against CD28 or CTLA-4 (data not shown). All killing of the bone marrow–derived macrophages and dendritic cells appeared to be mediated through the perforin pathway, since LAK cells from perforin$^{-/-}$ mice did not lyse these cells (data not shown).

Discussion

Using purified populations of mouse NK cells, we demonstrate that the B7-1 gene product functions as a triggering signal for NK cell–mediated cytotoxicity. The strength of the triggering response is such that it overrides the protection mediated by MHC class I molecules. Triggering of NK cells by B7-1-transfected target cells appears not to be dependent on the CD28 or CTLA-4 receptors. In addition to B7-1-transfected tumor cell lines, we demonstrate that bone marrow–derived macrophages and dendritic cells are highly susceptible to NK cell–mediated lysis despite expression of MHC class I molecules.

The general belief holds that NK cell–mediated cytotoxicity is controlled by positive as well as negative signals. While the latter have attracted much attention during the last years, significantly less is known about the former (Raulet, 1996). The present data strongly suggest that costimulatory molecules, such as B7-1, may fall into the category of NK cell–triggering molecules. The consensus has been that the negative signal provided by MHC class I molecules dominates over triggering signals (e.g., see Correa et al., 1994). The present results suggest that some triggering responses readily override the inhibitory signals provided by MHC class I molecules. We propose that the final outcome of NK cell–mediated cytotoxicity is a result of the interaction between triggering and inhibitory ligands and receptors, respectively, and that the net result of these interactions will determine the susceptibility to lysis. In the present model, focusing on the interplay between one triggering (B7-1) and one inhibitory (MHC class I) ligand, target cell susceptibility varies according to the following equation, with the most NK-resistant cells mentioned first: MHC class I$^+$/B7-1$^-$ < MHC class I$^-$/B7-1$^-$ = MHC class I$^+$/B7-1$^+$ < MHC class I$^-$/B7-1$^+$.

The present results indicate that the expression of B7-1 on tumor cells per se triggers NK cells and that this is not the consequence of indirect events, such as concomitant down-regulation of MHC class I. The NK cell–triggering ligands on mouse bone marrow–derived macrophages and dendritic cells are unknown. However, since they do express significant levels of costimulatory molecules such as B7-1 and B7-2 (Linsley and Ledbetter, 1993; Allison, 1994; Sharpe, 1995), it is tempting to speculate that these molecules may contribute to some extent to the NK cell–sensitive phenotype. Lysis of both B7-1$^+$ tumors and APC required perforin, suggesting that Fas–FasL interactions would have no role,

or only a minor role, in these types of cytotoxic responses. This differs from results with the human YT2C2 cell line, in which the Fas–FasL pathway contributed, at least in part, to the lytic activity (Montel et al., 1995b).

T lymphocyte recognition of B7-1 leads to either T cell activation or inhibition depending on whether the interaction is mediated by CD28 or CTLA-4 (Linsley and Ledbetter, 1993; Allison, 1994; Sharpe, 1995; Tivol et al., 1995; Waterhouse et al., 1995). We have shown that B7-1 molecules trigger NK cell cytotoxicity. However, B7-1 molecules may impose other effects on NK cells as well, including the induction of NK cell proliferation and cytokine secretion (Nandi et al., 1994). Our results further indicate that the receptors on NK cells that recognize B7-1 may be different from CD28 and CTLA-4, or at least not restricted to these receptors. This contrasts with the results obtained with the human NK cell leukemia YT2C2 line, where B7-1 triggering was mediated through interactions with CD28 (Azuma et al., 1992; Montel et al., 1995a). The physiological significance of the latter findings is less clear, however, because adult human NK cells are CD28 negative.

The transfection of B7-1 into tumor cell lines has become an attractive means for anti-tumor immunotherapy to induce strong anti-tumor cytotoxic T cells. It is believed that B7-1-expressing tumor cells may function directly as professional APC, being capable of activating naive T cells. The present data, as well as previous findings (Cavallo et al., 1995; Geldhof et al., 1995; Wu et al., 1995; Yeh et al., 1995), suggest that B7-1-transfected tumor cells may serve as targets for NK cells. These observations have led to a reinterpretation of the role of costimulatory molecules in anti-tumor immunity (Cavallo et al., 1995; Wu et al., 1995). In light of the present data, we speculate that target cells transfected with costimulatory molecules may, at least in part, be destroyed by NK cells. Cellular debris from tumor cells destroyed by NK cells could be processed by professional APC, which will then present tumor antigen on MHC molecules. This event would in turn activate naive T cells, leading to the anti-tumor specific responses.

There have been previous observations regarding the ability of NK cells to interfere with lymphocyte proliferation in mixed lymphocyte cultures. In those studies, it was speculated that NK cells might exert this mechanism of action by interfering with APC such as dendritic cells (Gilbertson et al., 1986). The present findings indicate, but do not prove, a role for NK cells in the control of immune responses involving activated APC. We have shown that autologous bone marrow–derived APC can efficiently be lysed by NK cells. This event could control macrophages or dendritic cells in their stimulation of naive T cells in lymphoid organs or ensure that these cells do not end up in the periphery and cause inflammatory or other unwanted responses. Further studies may provide insights into the physiological role of NK cell–mediated triggering by costimulatory molecules such as B7-1 and NK cell interaction with APC.

Experimental Procedures

Cell Lines and Antibodies

EL-4 is a benzpyrene-induced T cell lymphoma of C57BL/6 (B6) origin. C4.4-25$^-$ is an MHC class I–deficient (β2-microglobulin$^-$ [β2m$^-$]) variant of EL-4 (Glas et al., 1992). RMA is a variant of the Rauscher virus–induced T cell lymphoma RBL-5 of B6 background. RMA-S is an MHC class I–deficient (TAP2-deficient) variant of RMA (Ljunggren and Kärre, 1985). YAC-1 is a highly NK cell–sensitive Moloney virus–induced T cell lymphoma of A/Sn background. P815 is a mastocytoma of DBA/2 background. EL-4 cells transfected with vectors containing the murine B7-1 gene (Chen et al., 1994) and vector control cells were provided by Drs. T. Wen and I. Hellström. B7-1 transfectants of RMA, RMA-S, and P815 (designated RMA B7-1, RMA-S B7-1, and P815 B7-1, respectively) were generated by electroporation using a B7-1 construct in a pBR322 plasmid (a gift to Dr. K. Kärre from Dr. G. J. Freeman, Dana–Farber Cancer Institute and Harvard Medical School, Boston, MA). All cells, including vector controls, were screened on a regular basis for B7-1 expression by flow cytometry with the biotinylated anti-CD80 (B7-1) monoclonal antibody 16-10A1 (Pharmingen, San Diego, CA) and Neutralite avidin–FITC (Southern Biotechnology Associates, Birmingham, AL). For blocking studies, hamster anti-CTLA-4 (UC10-4F10-11) and anti-CD28 (37.51) were used (both from Pharmingen). The CTLA-4Ig fusion protein was provided originally by Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland). For fluorescence-activated cell sorter (FACS) analysis of the effector populations, biotin-conjugated anti-Ly-49A (A1), anti-TCR$\alpha\beta$ (H57-597), anti-CD8 (53-6.7), FITC-conjugated anti-Ly-49C (SW5E6), and PE-conjugated anti-NK1.1 (PK136) were used (all from Pharmingen). For assessment of MHC class I expression, FITC-conjugated anti-H-2K^b (AF6-88.5) and anti-H-2D^b (KH95) were used (both from Pharmingen).

Mice

B6 mice were from B&K Universal AB (Sollentuna, Sweden) and from the Microbiology and Tumor Biology Center of the Karolinska Institute (Stockholm, Sweden). β2m$^{-/-}$ (Koller et al., 1990), CD28$^{-/-}$ (Shahinian et al., 1993), and perforin$^{-/-}$ (Kägi et al., 1994) mice have been described previously. All mice were maintained at the Microbiology and Tumor Biology Center of the Karolinska Institute. The mutant mice were gifts of Drs. B. H. Koller, T. W. Mak, and H. Hengartner. Animal care was in accordance with institutional guidelines.

NK Cell–Mediated Cytotoxicity

Two different protocols for activation of NK cells were used. For activation of NK cells by interleukin-2 (IL-2), 25×10^6 spleen cells were resuspended in 10 ml of complete medium (αMEM, 10 mM HEPES, 2×10^{-7} M 2-mercaptoethanol, 10% FCS; all reagents from GIBCO Laboratories, Uxbridge, UK) and 1000 U/ml rIL-2 and cultured in 25 cm^2 tissue culture flasks in 10% CO_2 at 37°C. These effector cells are referred to as LAK cells. After 4 days, nonadherent and adherent cells were removed, washed once, and resuspended in complete medium without rIL-2 and then used as effectors. In antibody blocking studies with anti-CD28 or anti-CTLA-4 antibodies, effectors were preincubated with 50 μg, 10 μg, 2 μg, and 0.4 μg of antibody for 30 min at room temperature or at 37°C before the cytotoxicity assay. For blocking with CTLA-4Ig, 20 μg/ml was added to target cells for 30 min at room temperature or at 37°C before the cytotoxicity assay. For both of these blocking studies, antibodies and the fusion protein were present during the cytotoxicity assay. For isolation of NK1.1$^+$, CD8$^+$, TCR$\alpha\beta^+$, Ly-49A, and Ly-49C single or double positive effectors, rIL-2-activated splenocytes cultured for 4 days (see above) were labeled for flow cytometry. Sorting of cells was performed on a FACS Vantage cell sorter (Becton Dickinson, Mountain View, CA). Sorted cells were cultured in IL-2-containing complete medium for 1 additional day to dissociate the antibodies employed for sorting and then used as effectors. For activation of NK cells with the interferon inducer Tilorone (T-8014) (Sigma, St. Louis, MO), 0.2 ml of a 10 mg/ml solution of Tilorone was given per os 24 hr before sacrifice and removal of the spleen. Single cell suspensions of splenocytes depleted from erythrocytes by osmotic lysis were used as effector cells. All cytotoxicity studies were performed in standard 4 hr ^{51}Cr release assays.

Generation of Bone Marrow–Derived Macrophages and Dendritic Cells

Bone marrow–derived macrophages from B6 mice (unless stated otherwise) were expanded in L-cell supernatants as described previously (Racoosin and Swanson, 1989). After 8–10 days in culture,

these cells were used as targets in a standard ^{51}Cr release assay. Resting macrophages were obtained by flushing the peritoneal cavity of B6 mice. The erythrocytes were lysed by osmotic shock, and the remaining cells were incubated for 1 hr at 37°C in plastic dishes. The nonadherent cells were removed, and the remaining adherent cells were harvested and used as macrophages. Bone marrow–derived dendritic cells were obtained from B6 mice (unless stated otherwise) using the protocol of Inaba et al. (1992) with the following alterations. Bone marrow cells were cultured in Dulbecco's modified Eagle's medium containing 10% supernatant from the GM-CSF-secreting cell line X63 (a gift from Dr. C. Watts, University of Dundee, Dundee, UK) and 20% FCS. The culture medium was replaced every third day, and the cells were replated on day 7. On day 8, the nonadherent cells were used as targets in a standard 4 hr ^{51}Cr release assay.

Generation of ConA-Activated T Cell Blasts

For the generation of ConA-activated T cell blasts, 10^7 spleen cells depleted from erythrocytes were cultured in RPMI 1640 supplemented with 10% FCS and 1.5 μg/ml ConA (Sigma) for 48 hr and then used as targets in a standard 4 hr ^{51}Cr release assay.

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